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THE IDENTIFICATION OF THREE PREVIOUSLY UNREPORTED LINDANE METABOLITES FROM MAMMALS

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ABSTRACT

Previously unreported metabolites from the urine of rats treated with lindane have been identified as configurational isomers of 2,4,5,6- and 2,3,4,6-tetrachloro-2-cyclohexen-1-ol. In addition, an intermediate metabolite from the incubation of lindane with liver preparations, under N_2, has been identified as the configurational isomer γ-3,4,5,6-tetrachlorocyclohex-1-ene. The pathways leading to these metabolites appear to have an important role in the metabolism of lindane by mammals.

INTRODUCTION

While isolating and identifying five unreported urinary metabolites of γ-hexachlorocyclohexane (lindane),[1] small amounts of two other metabolites were detected. Though these metabolites were eventually isolated, identified and reported in a private communication,[2] publication of the data was deferred due to the low level and apparent unimportance of the metabolites in urine from rats fed diets containing 400 ppm lindane. Recent work, however, indicates that these metabolites actually constitute a significant proportion of the excreted lindane metabolites. Their isolation and characterization is described in this paper. In addition, an intermediate metabolite from the incubation of lindane with liver homogenates, under N_2, was identified as γ-3,4,5,6-tetrachlorocyclohex-1-ene (γ-TCCH). Though γ-TCCH has previously been reported as a lindane metabolite from microorganisms,[3-8] there has been no evidence to date that mammals produce this intermediate. However, recent work indicates that γ-TCCH is probably an intermediate in the metabolism of lindane to CO_2[9] and 4-chlorophenylmercapturic acid.[10]

EXPERIMENTAL

Weanling female Sprague-Dawley rats individually housed in metabolism cages were fed diets containing 400 ppm lindane (obtained from the Health Effects Research Laboratory Analytical Reference Standards Repository, Research Triangle Park, North Carolina). For the next month, the urine samples were pooled daily, acidified to pH 2.0 and extracted 3 times with equal volumes of hexane. The combined hexane extracts were then partitioned with a volume of 0.15 N sodium hydroxide equivalent to 25% that of the organic phase to remove the chlorophenols.

After chromatography on an activated silicic acid column as previously described,[1] the fractions containing the two unidentified metabolites and 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol (PCCOL) were concentrated by flash evaporation. The metabolites were separated on two silica gel 60 pre-packed columns, size A (obtained from EM Laboratories, Inc., Elmsford, New York), placed in series. After the columns were flushed with benzene for 60 minutes, 0.5 ml of the concentrated metabolite fraction was injected and the columns were eluted with benzene. One hundred 1 ml samples were collected. The metabolite tentatively identified as 2,4,5,6-tetrachloro-2-cyclohexen-1-ol was eluted in fractions 79-90, that identified as 2,3,4,6-tetrachloro-2-cyclohexen-1-ol was eluted in fractions 55-65 and PCCOL was recovered from fractions 25-40. These metabolites were further purified by collection of the effluent, at the appropriate retention time, during gas chromatography of the extracts. The metabolites were collected in sufficient quantity and purity to prepare the acetate derivatives[11] and obtain unadulturated NMR and mass spectra.

Hydrolysis of the glucuronide conjugates of the alcohol isomers was carried out using bacterial β-glucuronidase (Type V-A from Sigma Chemical Co., St. Louis, Missouri) and a procedure employed by Durston and Ames.[12]

The sulfate conjugates of the alcohol isomers were determined on 10 ml urine aliquots after 24 hours incubation with 0.4 U/sample of Aerobacter aerogenes aryl sulfatase (Sigma Chemical Co., St. Louis, Missouri). The urine aliquots for both glucuronides and sulfates were extracted twice with benzene and analyzed by g.l.c.

To determine the comparative urinary level of the alcohol isomers, 6 weanling female Sprague-Dawley rats were treated p.o. with 2 mg of lindane in 0.1 ml of peanut oil. Individual urines, collected for 24 hours following the lindane treatment, were extracted and analyzed by g.l.c.

In collecting the lindane metabolite, γ-3,4,5,6-tetrachlorocyclohex-1-ene, from liver homogenates for indentification, tissue preparation, incubation conditions and extraction procedures previously described[13] were used with the exception that N_2 was bubbled gently into the incubation flasks for 1 minute prior to incubation. The metabolite from the combined extracts was isolated by chromatography on an activated silicic acid column as previously described[1] and purified by collecting the effluent at the appropriate retention time during gas chromatography of the extract.

The catalytic dehydrogenation used in the characterization of the alcohol isomers involved a charcoal-palladium catalyst prepared by a standard procedure.[14] Each metabolite was dissolved in 1 ml of decalin with a small portion of the catalyst. The mixture was heated at 190°C for 5 hours, and then partitioned into 0.15 N NaOH. The NaOH was acidified, extracted with benzene and the benzene was analyzed by g.l.c.

The gas chromatograph employed was a Tracor Model MT 220 equipped with an electron capture detector and a Coulson electrolytic conductivity detector operated in the oxidative mode.

Other instruments employed for the identification included a Hewlett-Packard 5930A mass spectrometer equipped with a Model 5700A gas chromatograph and a Model 5932A data system together with a Varian T-60 NMR spectrometer operating at 60 MHz and 35°C.

RESULTS AND DISCUSSION

Rats fed diets containing 400 ppm lindane excreted two unknown neutral metabolites, M-1 and M-2 (TABLE 1), which had shorter GLC retention times on two different columns than the previously identified metabolite, 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol (PCCOL).[1] Moreover, like PCCOL, both M-1 and M-2 acetylated[11] to give products with longer retention times than PCCOL (TABLE 1).

TABLE 1

Retention Times of M-1, M-2, PCCOL and Their Acetate Derivatives

Metabolite Identification	Retention Time (min.)		RRT(c)	
	Column A(a)	Column B(b)	Column A(a)	Column B(b)
M-1	10.1	8.00	0.48	0.53
M-2	13.8	8.50	0.66	0.56
PCCOL	20.9	15.2	1.00	1.00
M-1 Acetate	24.4		1.17	
M-2 Acetate	31.0		1.48	
PCCOL Acetate	39.0		1.86	

Abbreviation: PCCOL, 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol
(a)5% OV-210 on 100/120 mesh Gas Chrom Q at 110°C.
(b)5% DEGS on 100/120 mesh Chromosorb W at 183°C.
(c)Retention times of PCCOL and unknowns relative to PCCOL.

Recently it was found that these metabolites were excreted predominately as glucuronide and sulfate conjugates (TABLE 2).

TABLE 2

Excretion of Free and Conjugated M-1, M-2, and PCCOL

Metabolite Identification	Free mg/24 hr	Glucuronides µg/24 hr	Sulfates µg/24 hr	Total µg/24 hr
M-1	0.46 ± 0.19	12.8 ± 2.30	0.45 ± 0.04	13.5 ± 2.40
M-2	-	0.42 ± 0.09	0.04 ± 0.002	0.51 ± 0.10
PCCOL	0.30 ± 0.09	23.5 ± 4.50	0.24 ± 0.13	24.0 ± 4.40

Abbreviation: PCCOL, 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol
Values are means ± SE. Treatment is described in Experimental.

These conjugates were not detected immediately due to the acid hydrolysis procedure routinely used to free the chlorophenol conjugates.[15] This procedure results in the degradation of M-1, M-2, and PCCOL and, thus, these metabolites were never detected in the extract of the hydrolyzed urine. In an attempt to separate the glucuronide and sulfate conjugates of the chlorophenols, urine was incubated for 24 hours at 37°C with β-glucuronidase[12] or with sulfatase as described in Experimental. As indicated in Table 2, recovery of M-1, M-2, and PCCOL increased to such an extent that they rival the chlorophenols in importance as lindane metabolites.

The mass spectra of both M-1 and M-2 have a molecular ion at 234 a.m.u. that showed a 4 Cl isotope cluster (Figs. 1 and 2). However, the intensity of this molecular ion was 30 times greater for M-2 (Fig. 2) than for M-1 (Fig. 1). The molecular ion assignment was further

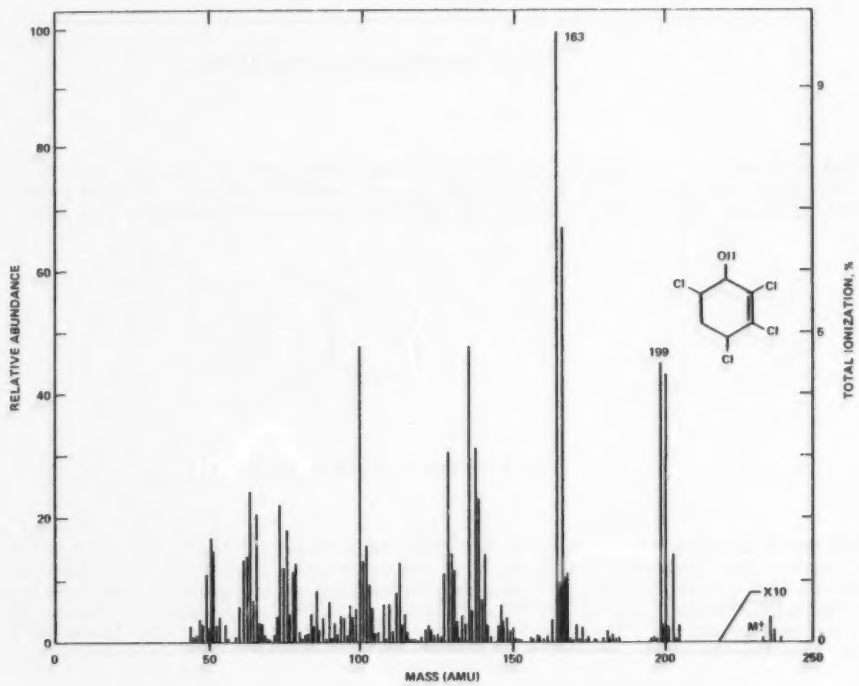

Fig. 1. Mass spectrum of metabolite M-1.

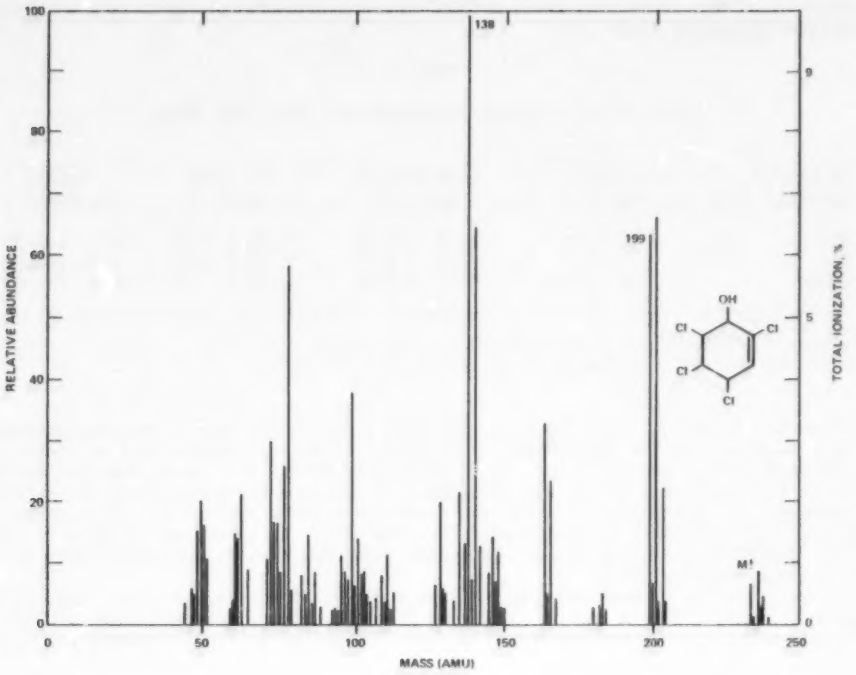

Fig. 2. Mass spectrum of metabolite M-2.

supported by intense ion fragments at 199 a.m.u. that represent the logical neutral loss, M-Cl. These fragments contained a 3 Cl isotope cluster in both spectra. At this point in the identification, any isomer of the following structure could accommodate the data. However,

Fig. 3. Possible structures for M-1 and M-2.

dehydrogenation of both M-1 and M-2, as described in Methods, yielded only 2,3,4,6-tetrachlorophenol. This indicated the location of the chlorines on the ring. For example, if Cl were absent from C-2 or C-6 (Fig. 4) then dehydrogenation would result in the formation of 2,3,4,5-tetrachlorophenol whereas if Cl were absent from C-4, dehydrogenation would yield 2,3,5,6-tetrachlorophenol. These results indicate that M-1 has one of the following structures while M-2 has the other.

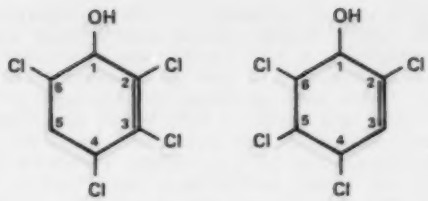

Fig. 4. Possible structures for M-1 and M-2.

The mass spectrum of M-2 (Fig. 2) had an intense 2 Cl isotope cluster at 138 a.m.u. This fragment corresponded to the loss of 96 mass units and was consistent with the elimination of dichloroethylene, M-[$C_2H_2Cl_2$]. This fragmentation was consistent with the retro-Diels-Alder elimination illustrated in Figure 5 and permitted the tentative identification of M-2 as structure II in Figure 4, a configurational isomer of 2,4,5,6-tetrachloro-2-cyclohexen-1-ol (2,4,5,6-TCCOL). Such fragmentations have been well documented for cyclohexenes[16] as well as for the related lindane metabolite PCCOL.[1]

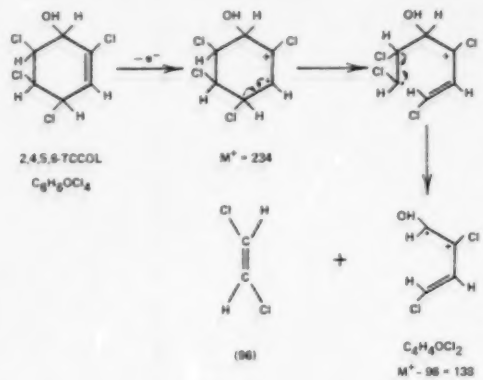

Fig. 5. Retro-Diels-Alder Elimination of dichloroethylene from 2,4,5,6-TCCOL.

The mass spectrum of M-1 (Fig. 1) was dominated by a 163 a.m.u. ion that contained a 2 Cl isotope cluster. This fragment resulted from the composite loss of a Cl atom and HCl, M-[Cl + HCl]. While the fragmentation pattern for M-2 was characterized by a retro-Diels-Alder

elimination (Fig. 3), there was no evidence for the analogous elimination of chloroethylene in the mass spectrum of M-1. Other fragmentations predominated in the case of M-1. A fragment observed at 171 a.m.u. containing a 3 Cl cluster was consistent with the loss of a Cl atom and CO, M-[Cl + CO] and was analogous to a similar fragmentation observed in the mass spectrum of PCCOL.[1] Small neutral losses were observed in overlapping fragments at 181 and 180 a.m.u. corresponding to M-[Cl + H_2O] and M-[HCl + H_2O] which were consistent with the molecular ion assignment and the alcohol nature of the molecule. Thus, M-1 was tentatively identified as 2,3,4,6-tetrachloro-2-cyclohexen-1-ol (2,3,4,6-TCCOL, structure I in Figure 4).

The NMR spectrum of the isomer tentatively identified as 2,3,4,6-TCCOL exhibited multiplets centered at 4.5 δ (2H) for H-4 and H-6; 2.5 δ (1H) for H-1; and 1.2 δ (3H) for H-5 and -OH. The NMR spectrum of 2,4,5,6-TCCOL showed multiplets centered at 4.4 δ (3H) for H-4, H-5, and H-6; 2.9 δ (2H) for H-1 and H-3, and a broad singlet at 2.5 δ for the OH proton. The presence of the CH_2 resonance for the 2,3,4,6-TCCOL isomer and the absence of this resonance for the 2,4,5,6-TCCOL isomer and the presence of 3 saturated carbons each bearing a geminal proton and chlorine for the 2,4,5,6-TCCOL isomer corroborates the assignment of structures having a vinylic hydrogen at the 3 position to the 2,4,5,6-TCCOL isomer and a vinylic chlorine at this position to 2,3,4,6-TCCOL.

When lindane, together with an NADPH generating system, is incubated at 37°C under N_2, a chlorinated nonpolar metabolite is formed. The mass spectrum for this metabolite had a molecular ion at 218 a.m.u. which contained a 4 Cl isotope cluster (Fig. 6). This molecular ion assignment was supported by overlapping fragments at 183 and 182 a.m.u. corresponding to M-[Cl] and M-[HCl], respectively. The base peak at 147 a.m.u. contained a 2 Cl isotope cluster

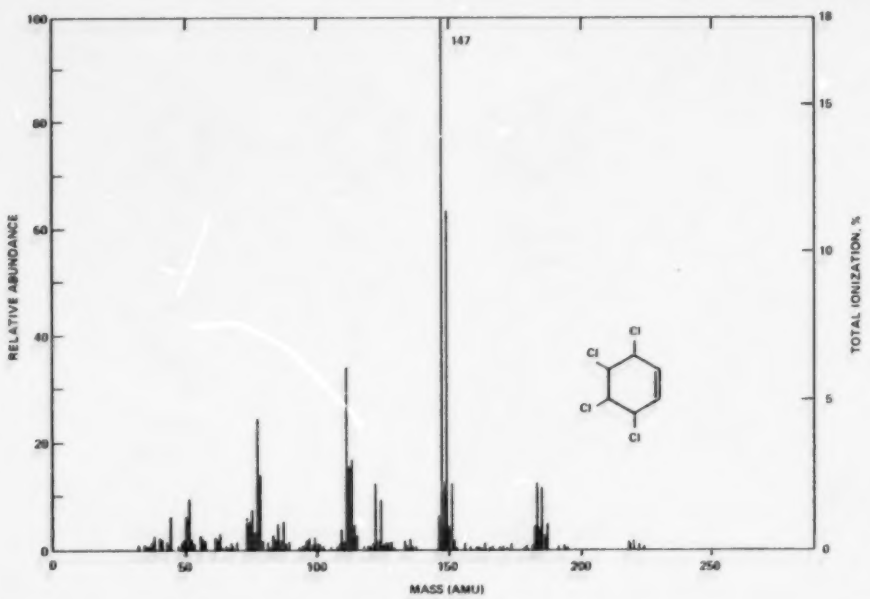

Fig. 6. Mass spectrum of intermediate metabolite.

and represented the loss of a Cl atom and HCl, M-[Cl + HCl]. Additional fragments included M-[Cl + 2 HCl] at 111 a.m.u. and the overlapping M-[2 Cl + HCl] at 112 a.m.u. The fragment at 122 a.m.u. containing a 2 Cl isotope cluster resulted from a retro-Diels-Alder elimination of dichloroethylene, M-[$C_2H_2Cl_2$]. On the basis of the mass spectra, the metabolite was tentatively identified as a configurational isomer of 3,4,5,6-tetrachlorocyclohex-1-ene (TCCH). Authentic samples of α-, β-, γ-, δ-, and ε-3,4,5,6-TCCH were obtained from Dr. N. Kurihara,

Fig. 7. Configurational isomers of 3,4,5,6-tetrachlorocyclohex-1-ene.

Kyoto University, Kyoto, Japan (Fig. 7)and these were compared to the metabolite by GLC chromatography on 2 different columns (TABLE 3).

TABLE 3

Metabolite Identification	Retention Time (min.)		RRT[c]	
	Column A[a]	Column B[b]	Column A[a]	Column B[b]
γ-HCH	10.2	11.5	1.00	1.00
α-TCCH	1.53	1.15	0.15	0.10
β-TCCH	2.75	3.22	0.27	0.28
γ-TCCH	2.96	3.45	0.29	0.30
δ-TCCH	2.14	2.30	0.21	0.20
ε-TCCH	9.59	11.2	0.94	0.97

(a)1.5% OV-17 + 1.95% OV 210 on 80/100 mesh Gas Chrom Q at 165°C.
(b)5% DEGS on 80/100 mesh Gas Chrom P at 165°C.
(c)Retention time relative to lindane (γ HCH).

The metabolite had identical retention times on both GLC columns to that of the γ-TCCH isomer and this fact together with the mass spectral data was considered sufficiently conclusive to permit the tentative identification of the neutral intermediate metabolite as γ-3,4,5,6-tetrachlorocyclohex-1-ene.

This intermediate metabolite has been implicated in the metabolism of lindane to CO_2[5],[9] and to 4-chlorophenylmercapturic acid.[10]

With the discovery that the TCCOL isomers were more than 95% conjugated (TABLE 2), there was a corresponding increase in the recovery of these metabolites and PCCOL from the urine. The level of the alcohol isomers found in the urine of rats treated with a single 2 mg dose of lindane amounts to a substantial 25% of the total urinary chlorophenols excreted by these animals. Moreover, there is some evidence that the alcohol isomers may be metabolites of lindane in mould cultures.[17]

In summary, the identification of 3 unreported lindane metabolites has demonstrated the existence of some important and novel routes to the degradation of this pesticide in the environment.

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